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DIAGNOSIS OF (AIDS) RELATED INTESTINAL PARASITES

ANNUAL REPORT

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FOREWORD

In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources Commission on Life Sciences, National Research Council (DHHS, PHS, NIH Publication No. 86-23, Revised 1985).

For the protection of human subjects the investigator(s) have adhered to policies of applicable Federal Law 45CFR46.

I. BACKGROUND AND STATEMENT OF THE PROBLEM

Persistent diarrhea from infection with the protozoan parasite Cryptosporidium, continues to be an indicator illness of Human Immunodeficiency Virus (HIV) infection. During the past year, our understanding of the dimensions and import of Cryptosporidium infection in this patient population, and in others, has continued to expand. From an epidemiologic perspective: 15% of patients with AIDS and diarrhea recently evaluated at the National Institutes of Health had cryptosporidiosis (1) while 16% of patients with AIDS and diarrhea studied at Johns Hopkins were infected with Cryptosporidium, the most common pathogen isolated in this series (2). At St. Stephen's Hospital in Great Britain, 11% of AIDS patients had Cryptosporidium infection and 19% of these were thought to have died as a direct result (3); 12% of AIDS patients reported from Sao Paulo, Brazil, also had cryptosporidiosis (4).

From a clinical perspective: Fulminant diarrhea and concomitant malabsorption, dehydration and electrolyte imbalances remain the major causes of morbidity and mortality from cryptosporidiosis. However, in addition, Cryptosporidium is now recognized, particularly in HIV-infected patients, as a cause of acalculous cholecystitis, cholangitis, hepatitis, pancreatitis and a variety of respiratory problems (5-10). Infection with Cryptosporidium remains highly refractory to therapeutic intervention, with treatment limited to hydration and hyperalimentation. Although close to eighty chemotherapeutic, biologic and other anti-diarrheal agents have now been evaluated, only anecdotal success has been reported. In at least three trials, spiramycin, previously the most promising agent, has now been shown to have little predictable efficacy (3,11,12). Bovine-derived products, especially colostrum hyperimmune to Cryptosporidium, have been tried recently in both animals and humans, and may be protective and/or therapeutic (13,14,15,16). Other agents which have been proposed for future human study include Octreotide Acetate or somatostatin and Diclazuril Sodium, a veterinary product produced and used in Europe for treatment of related infections in animals. Lack of a simple in vitro cultivation system or a suitable small animal model continue to hamper the more rapid development and testing of interventional agents as well as definition of the specific immune deficits which permit prolonged Cryptosporidium infection in HIV-infected persons.

In other populations, particularly in less developed areas of the world, Cryptosporidium is now thought to be a leading enteropathogen. More than 75 geographically-based surveys of fecal excretion of Cryptosporidium oocysts have shown mean prevalences in selected groups in the less developed world which range from 4.9% in Asia to 9.4% in Africa (summarized from 33 references, book chapter in preparation). Infection can be severe and debilitating for as long as thirty days in immunologically healthy individuals, with potential for transmission persistent for an additional sixty days after symptoms remit but while infectious oocysts are still shed (17,18). Of particular relevance are several groups now thought to be at increased risk for Cryptosporidium infection: Nosocomial spread of Cryptosporidium to hospitalized patients has been noted (12,19), and immunologically healthy family members and health care workers are frequently infected after contact with a patient with cryptosporidiosis (20,21,22,23). Travellers or temporary workers in areas of high endemicity are also at increased risk (24,25,26,27,28).

Although ability to diagnose Cryptosporidium infections rapidly and accurately has changed little in the past year and remains subject to the proficiency of the individual laboratory, priorities may more logically begin to focus on successful prophylactic and therapeutic intervention in Cryptosporidium infection.

II. RESULTS TO DATE

A. Development of an ELISA to detect Cryptosporidium antigens in fecal specimens.

A double antibody indirect ELISA test has now been developed which detects Cryptosporidium antigens in fecal specimens. Reagents for this assay were produced by: (1) oral infection of colostrum - deprived calves with Cryptosporidium and collection of fecal output (USDA collaboration); (2) purification of Cryptosporidium oocysts from calf feces for use as immunogens by flotation in distilled water saturated with sodium chloride, with subsequent sedimentation in distilled water, washing in sodium hypochlorite, and sonication; (3) immunization of rabbits and goats, screened negative for specific anti-Cryptosporidium antibodies, with purified Cryptosporidium over an eight-month period to produce high-titered specific antisera. Optimal antibody combinations as well as concentrations for the ELISA test were then determined, by checkerboard titration using either purified Cryptosporidium oocysts or Cryptosporidium oocysts in calf feces as antigen.

The following ELISA procedure was established using standard incubation times and washing procedures. ELISA microtiter plates were prepared by coating alternate double rows of wells with 100 µl of 1 1:10,000 dilution of either immune or non-immune rabbit antisera in carbonate buffer (pH 9.6). Plates were stored at 4°C for at least 14 hours before use. Test material was prepared by mixing 25 µl of antigen (feces homogenized in phosphate buffered saline [PBS]) with 75 µl of PBS-Tween20 - 0.5% gelatin (PBS-T-G). This was added to 4 wells, two coated with immune and two with non-immune rabbit antisera, which ultimately minimizes the role of non-specific color reactivity. Immune goat antisera diluted 1:400 in PBS-T-G was the second antibody used, and commercial enzyme-conjugated anti-goat immunoglobulin and substrate completed the assay.

When fecal specimens were tested, at least three negative and one positive control samples were applied to each plate to allow standardization between microtiter plates. To calculate results, the mean optical density (O.D.) reading of wells coated with non-immune rabbit sera was subtracted from the mean of those coated with immune sera to give a specific O.D. for a clinical specimen. The mean and standard deviation for the negative control specimens were calculated as above and a clinical specimen was considered positive if its specific O.D. value was greater than the mean O.D. plus 2 standard deviations of the negative controls.

The ELISA detected between 2000 and 4000 purified Cryptosporidium oocysts. Initial testing using a modest number of fecal specimens from HIV-infected individuals suggested utility for the assay clinically. To establish sensitivity and specificity most expeditiously, in collaboration with investigators from Universidad Peruana Cayetano Heredia, Lima, Peru, and the Johns Hopkins University School of Hygiene and Public Health, arrangements were made for collection of fecal specimens over a six-month period from residents in an urban slum area near Lima, highly endemic for Cryptosporidium infection.

From one to eight specimens were collected on each of 106 individuals for a total of 230 specimens. These were examined locally using three different microscopic techniques for Cryptosporidium oocyst identification. Additionally, an aliquot of each specimen was immediately frozen at -70°C and preserved in 10% formalin. The ELISA test was set up both in Peru and in our own laboratories. Frozen specimens were each tested by ELISA between two and ten times. All specimens for which there was discrepancy between microscopic and ELISA examinations and all specimens where there was not concordance between initial microscopic examinations were re-examined in our laboratory using formalin-preserved aliquots.

Preliminary analysis of the Peruvian data shows that the over-all sensitivity of the assay was 80.4% and the over-all specificity was 97.7%. The sensitivity increases if fecal specimens which have never been thawed are tested. Some microscopy-positive ELISA-negative specimens had fewer than 5 Cryptosporidium oocysts detected microscopically on a single slide prepared from a concentrated fecal specimen which appears below the sensitivity of the assay and which may, in fact, not represent significant infection. 120 fecal specimens contained other intestinal parasites: Giardia lamblia (65), Endolimax nana (35), Entamoeba coli (33), Chilomastix mesnili (25), Ascaris lumbricoides (8), Hymenolepis nana (7), Trichuris trichiura (4), and Iodamoeba butschlii (3). There was no cross-reactivity with antigens in any of these specimens.

This ELISA to detect Cryptosporidium antigens in fecal specimens offers a simple, rapid, easily standardized diagnostic and epidemiologic screening tool. Future work may include use of this test in Zambia, although surveys by microscopic examination for Cryptosporidium oocyst shedding in a variety of populations would be a necessary prerequisite.

B. Use of Bovine Colostrum Hyperimmune to Cryptosporidium to prophylax against or to treat Cryptosporidium infection.

Bovine colostrum specifically hyperimmune to Cryptosporidium was produced by parenteral injection and intramammary infusion of oocysts into pregnant dairy cows (USDA collaboration). The hyperimmune state was indicated by titers > 1:200,000 of Cryptosporidium bovine immunoglobulins (IgG-1, IgM and IgA) detected by ELISA in our laboratory. This colostrum has been used to assess protective efficacy in neonatal calves: calves fed hyperimmune colostrum prior to oral infection with Cryptosporidium had significantly less diarrhea ($p < 0.02$) and shed oocysts for significantly less time ($p < 0.05$) than the control group (15).

The colostrum has also been used compassionately to treat a 38 year-old HIV-infected homosexual male with devastating cryptosporidiosis. The patient excreted 6 to 12 liters of stool per day for at least three months, two documented in hospital. Repeat stool examinations identified no bacterial or parasitic pathogen other then Cryptosporidium. Trials with more than six anti-diarrheal medications, including spiramycin, were ineffective. The patient received the hyperimmune colostrum by direct duodenal infusion, and during infusion, the patient's fecal output decreased to less that two liters per day.

Forty-eight hours after treatment, stools were fully formed and oocysts to Cryptosporidium could not be found. The patient remained asymptomatic for three months after treatment (16). These studies indicate that there is a biologically active factor(s) in hyperimmune bovine colostrum which needs to be further characterized.

C. Development of Mouse Models of Chronic Symptomatic Cryptosporidiosis.

Recently we have developed two new mouse models for chronic symptomatic cryptosporidiosis of the gastrointestinal and hepatobiliary tracts (manuscript in preparation). In the first of these, adult athymic nude mice (nu/nu) are orally infected with Cryptosporidium oocysts. They develop clinical symptoms as well as elevated oocyst shedding by the fourth week. The clinical syndrome mimics that seen in may HIV-infected patients: diarrhea waxes and wanes, as do periods of more and less intense oocyst passage. Animals have been followed for close to 20 weeks and, at necropsy of late-stage infected animals, Cryptosporidia have been identified throughout the gastrointestinal tract, as well as in the bile ducts, gallbladder, hepatic and pancreatic ducts, similar to the histopathology found in some HIV-infected patients. BALB/c (nu/nu) mice immunologically reconstituted with lymph node and spleen cells from previously infected BALB/c (nu/+) animals were able to clear their Cryptosporidium infections.

In the second animal model, suckling BALB/c mice are orally infected with Cryptosporidium oocysts, but infection is prolonged by weekly administration of a specific monoclonal antibody (GK 1.5; rat IgG2b anti-mouse CD4) which deletes $CD4^+$ ($L3T4^+$) T cells. Deletion of the $CD8^+$ ($Lyt2^+$) T cell subset with another monoclonal antibody (2.43; rat IgG2b anti-mouse CD8), in contrast, fails to prolong neonatal infection. Clinical infection, oocyst shedding and necropsy findings are similar to those described for the athymic model as long as $CD4^+$ T-cell ablation continues, and discontinuation of monoclonal antibody administration allows clearance of Cryptosporidium organisms.

These animal models help define the T cell subset important in eradication of Cryptosporidium infection. They will be useful in further defining defects in the immune response to Cryptosporidium which might be amenable to immunotherapy, as well as in assessment of potential prophylactic and treatment modalities.

D. Publications and Abstracts

Publications

Treatment of cryptosporidiosis with hyperimmune bovine colostrum

1. Ungar BLP. Ward DJ, Fayer R and Quinn C: Successful Use of Hyperimmune Bovine Colostrum to Treat Cryptosporidium Infection in an Acquired Immunodeficiency Patient (manuscript submitted).
   Significance: Hyperimmune bovine colostrum has therapeutic benefit in an HIV-infected patient.

2. Fayer R, Andrews C, Ungar BLP and Blagburn B: Efficacy of Hyperimmune Bovine Colostrum for Prophylaxis of Cryptosporidiosis in Neonatal Calves. J Parasit (in press)
   Significance: Hyperimmune bovine colostrum has prophylactic benefit in calf model.

3. Moon H, Woodmansee D, Harp JA, Abel S and Ungar BLP: Lacteal Immunity to Enteric Cryptosporidiosis in Mice: Immune Dams do not Protect Their Suckling Pups. Infect Immun 56:649-653, 1988.
   Significance: Non-hyperimmune colostrum is not protective in mouse model.

Epidemiologic parameters of cryptosporidiosis

4. Ungar BLP, Mulligan M and Nutman TB: Serologic evidence of Cryptosporidium infection in U.S. Peace Corps Volunteers before and during Peace Corps Service in Africa. Arch Intern Med (in press)
   Significance: Immunologically healthy individuals based in endemic areas have a high rate of seroconversion and are likely at risk for infection.

5. Hayes EB, Matte TD, O'Brien TR, McKinley TW, Logsdon GS, Rose JB, Ungar BLP, Ward DM, Pinsky PF, Cummings ML, Wilson MA, Long EG, Hurwitz ES and Juranek DD: Contamination of a Conventionally Treated Filtered Public Water Supply by Cryptosporidium Associated with a Large Community Outbreak of Cryptosporidiosis. (manuscript submitted)
   Significance: Water-borne outbreak affecting approximately 13,000 individuals on a common water supply is described.

6. Ungar BLP, Gilman R, Lanata C and Perez-Schael I: Sero-epidemiology of Human Cryptosporidium Infection in Two Latin American Populations. J Infect Dis 157:551-556, 1988.
   Significance: Cryptosporidium has a higher prevalence in less developed areas of the world and persons in these areas are likely at risk for infection.

ELISA Testing for Entamoeba histolytica, Giardia lamblia, Cryptosporidium

7. Taylor DN, Houston R, Shlim DR, Echeverria P, Bhaibulaya M and Ungar BLP: The Etiology of Diarrheal Disease among Travelers, Foreign Residents, and Peace Corps Volunteers in Nepal. J Amer Med Assn 260:1245-1248, 1988.
   Significance: ELISA testing for Entamoeba histolytica antigen in fecal specimens is a viable alternative to traditional diagnostic techniques in specimens of diverse geographic origins.

Manuscripts in Preparation

Mouse Model of Chronic Cryptosporidiosis

8. Ungar BLP, Burris JA, Quinn CA, Finkelman FD: New Mouse Models for Chronic *Cryptosporidium* Infection in the Immunodeficient Host.
Significance: First development of a mouse model of symptomatic cryptosporidiosis which mimics illness seen in HIV-infected patients; this can be used to test interventional agents and to define specific immune defects leading to chronic illness.

ELISA Testing for *Entamoeba histolytica*, *Giardia lamblia*, *Cryptosporidium*

9. Martinez H, Ungar BLP, Miranda E, Vesteriqui M and Gilman R: Detection of *Giardia lamblia* Antigens by Enzyme Immunoassay in Peruvian Children Before and After Treatment.
Significance: ELISA testing for *Giardia lamblia* antigens in fecal specimens is a viable alternative to traditional diagnostic techniques even in field settings.

10. Ravdin JI Simjee AE, Petri WA, Murphy CF, Ungar BLP and Jackson TFHG: Comparison of Clinical Status, Zymodeme Analysis, and Serum Immunoblot Recognition of *Entamoeba histolytica* Antigens.
Significance: More sensitive reagents may provide a refined and more useful ELISA test to detect *Entamoeba histolytica* antigens in fecal specimens.

Abstracts

1. Treatment of cryptosporidiosis with hyperimmune bovine colostrum (submitted Fifth International Conference on AIDS, Montreal, 1989).

2. ELISA test to detect *Cryptosporidium* antigens in Fecal Specimens. BLP Ungar, CA Quinn (to be submitted to ICAAC, Houston, 1989).

3. Mouse model of chronic cryptosporidiosis (to be submitted to ICAAC, Houston, 1989)

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